

Turing cationic antimicrobial peptide KR-12 into Self-assembled nanobiotics with potent bacterial killing and LPS neutralizing activities

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Abstract

Gram-negative sepsis has become one of major increasing medical burdens globally, which is subjected to growing antibiotic resistance problem and the relatively delayed development of new antibiotics. LL-37, the only type of Cathelicidin identified in humans, has diverse biological activities including direct bactericidal action, regulation of inflammation and LPS-neutralization. The KR-12 peptide is the smallest portion of LL-37 with antibacterial action, which has been shown that could be modified into more effective antimicrobials. Here, we synthesize two myristoylated derivatives of KR-12, Myr-KR-12N and Myr-KR-12C, which can spontaneously form nanoparticles when mixed with deionized water. We show that myristoylated KR-12 derivatives possess a broad-spectrum and more powerful bactericidal activity through interrupting the membranes of bacteria. Myr-KR-12N rescues mice from lethal sepsis induced by *E. coli*, even more potent rescuing activity than meropenem. We also demonstrate that myristoylated KR-12 nanobiotic can significantly bind with LPS and inhibit the inflammation *in vitro* and Myr-KR-12N rescue mice from LPS-induced sepsis *in vivo*, even more potent rescuing activity than polymyxin B. Toxic experiments indicate that neither Myr-KR-12N nor Myr-KR-12C nanobiotics exhibits meaningful hemolytic activity, liver and kidney injury. We thus developed a novel nanobiotic with dual bactericidal and LPS-neutralization properties, which may provide good insights for clinical translation of antimicrobial peptides and the creation of new antibiotics.

1. Introduction

Gram-negative sepsis are results from an uncontrolled immune response to infection, in which immune cells are triggered by lipopolysaccharide (LPS) generated by the bacterial outer membrane, resulting in severe inflammation, organ failure, and even death[1]. It has become one of major increasing medical burdens globally and a major challenge for researchers and intensive care clinicians due to the high and growing occurrence and great genetic, pathophysiological, and clinical complexity[2]. The growing antibiotic resistance problem and the relatively delayed development of new antibiotics necessitate a rapid search for novel antimicrobials[3, 4].

Antimicrobial peptides (AMPs) are naturally occurring peptides having therapeutic potential in the innate immune system. They are short (12–100 amino acids), positively charged (net charge of 2–9), and amphiphilic peptides that have been isolated from single-cell bacteria, insects and other invertebrates, plants, amphibians, birds, fish, and mammals, including humans. AMPs represent the first line of host to defense against invading microorganisms[5]. Thousands of such peptides have been discovered and added to the antimicrobial peptide database[6]. Antibiotics are often bacteriostatic and target particular cellular processes, while AMPs are bactericidal and have several possible targets aside from their distinctive membrane disturbance[7]. What's more, AMPs were proved to be less susceptible to resistance compared with traditional antibiotic [8]. Besides, immune effects such as immunomodulation and regulation of microecological balance were also found in AMPs[7]. Due to their various activities, AMPs were thought to be effective antibiotic candidate. However, concerns with relatively modest bactericidal activity of AMPs have hampered their therapeutic development as it requires greater dosages for treatment and indicate more adverse effects.

The Cathelicidin family of AMPs has been identified in various mammalian species. LL-37, which is mainly expressed in epithelial cells and immune cells such as macrophages, dendritic cells, natural killer cells,

neutrophils, is the only type of Cathelicidin identified in humans. It has many different biological activities besides its direct pathogen killing activities, such as regulation of inflammation and lipopolysaccharide-neutralization[9, 10].

LL-37 shows modest antibacterial activity against a wide range of Gram-negative and Gram-positive bacteria, including *Pseudomonas*, *Escherichia*, *Staphylococcus*, and *Enterococcus* genera [11]. LL-37 can kill bacteria both directly and indirectly through antibacterial activity and immunomodulation. The major mechanism of action, like that of other AMPs, is membrane disruption. The smallest portion of LL-37 with antibacterial action was discovered as the KR-12 peptide (residues 18–29 of LL-37)[11, 12]. In comparison to parental LL-37, KR-12 has been demonstrated substantially stronger cell selectivity for bacteria over erythrocytes and preserved antiendotoxin action, making it an attractive candidate for novel antibiotics[13, 14].

Modification of members of the Cathelicidin family into antibacterial medicines with diverse functions has received a lot of attention in recent years. In rat models of septic shock, for example, a sheep myeloid antimicrobial peptide (SMAP)-29, a Cathelicidin-derived peptide, displayed antiendotoxin activity equivalent to polymyxin B and therapeutic effectiveness[15]. Lipopeptides derived from KR-12 were shown to be more efficient in killing bacteria *in vitro* and in treating MRSA infection in a mouse model *in vivo*[16]. Another study improved the hydrophobicity of a KR-12 analog, resulting in more effective bactericidal agents *in vivo*[17]. A previous study focused on the amino acid replacement and proved that substitutions showed differential effects against different organisms[18, 19].

Nanoscale structures have been created using noncovalent self-assembly of individual molecules for extensive biomedical applications using noncovalent self-assembly of individual molecules[20]. For example, utilizing nanotechnology, we effectively turned an AMP, human alpha defensin-5, into a powerful antibacterial agent [21]. Another study used nanotechnology to modify a mutant piece of LL-37, and the resulting micelles had significant antifungal activity. Furthermore, employing peptide-linked PLGA conjugate micelles, the anticancer effectiveness of an LL-37 peptide fragment analog was improved[22]. In this paper, we developed and synthesized a series of peptides based on KR-12 to test their treating activity and toxicities *in vitro* and *in vivo*. We produced a new nanobiotic that was found to be more efficient than meropenem in an *in vivo* mouse sepsis model and to be much more potent than polymyxin in neutralizing LPS. Our findings will aid in the creation of new antibiotics.

2. Results and discussion

2.1 Design and Characterization of Myristoylated KR-12

Hydrophobicity and positive charge are critical factors for determining the bactericidal activity of antimicrobial peptides (AMPs). Increasing the hydrophobicity of AMPs facilitates their self-assembly into nanostructures in aqueous solutions. This self-assembly process increases the local positive charge, which promotes the binding of the peptides to negatively charged bacteria, ultimately disrupting the outer membrane (OM) and inner membrane (IM) structures of the bacteria. Figure 1A illustrates the design concept. KR-12's hydrophobicity was improved to facilitate its nanoassembly in aqueous solutions since it is extremely soluble in water[7]. As a result, a myristic acid, a 14-carbon saturated fatty acid called myristic acid was attached to either its N- or C-

terminus. To ensure that the fatty acids do not affect the conformation of KR-12, three glycine (Gly) residues were incorporated as linkers between the peptide and fatty acid blocks. Gly was chosen as it does not contain an R motif. The N-terminus of KR-12 possesses a free amino group, allowing direct connection to myristic acid. However, the C-terminus of KR-12 lacks a free amino group. Therefore, in addition to the three Gly moieties at the C-terminus, a lysine (Lys) residue containing two free amino groups was attached. By increasing the local density of positive charge, nanoparticle formation is predicted to improve antibacterial capabilities[23]. KR-12 was chemically synthesized via solid-phase peptide synthesis (SPSS) and extended N-terminally by a Gly-Gly-Gly residue or C-terminally by a Gly-Gly-Gly-Lys linker, yielding two myristoylated derivatives of KR-12, Myr-KR-12N and Myr-KR-12C, respectively. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis confirmed the successful synthesis of the three peptides (Figure S1-S3), and all peptides were purified to a purity level of above 95% as determined by reversed-phase high-performance liquid chromatography (RP-HPLC) analysis (Figure S4-S6). The structures, molecular weights, and the theoretically calculated and measured molecular weights of the peptides are summarized in Table 1. The measured molecular weights of the peptides matched well with their theoretical values, indicating successful synthesis. The hydrophobicity differences among the peptides in aqueous solution were reliably reflected by their distinct RP-HPLC retention times. The retention times for KR-12, Myr-KR-12N, and Myr-KR-12C were 10.62, 14.22, and 12.93 min, respectively, suggesting the following hydrophobic order: KR-12 < Myr-KR-12C < Myr-KR-12N.

Table 1

The amino acid sequence and main physicochemical parameters of KR-12, Myr-KR-12N and Myr-KR-12C.

Peptide	Sequence ^a	Measured MW (Da) ^b	Theoretical MW (Da)	RT (min) ^c	Purity	Net charge
KR-12	KRIVQRIKDFLR-NH ₂	1572.05	1571.95	10.62	> 95%	+ 4
Myr-KR-12N	Myr-GGGKRIVQRIKDFLR-NH ₂	1952.12	1952.48	14.22	> 95%	+ 4
Myr-KR-12C	KRIVQRIKDFLRGGGK-Myr	2081.76	2081.65	12.93	> 95%	+ 5
^a Myr indicated myristic acid						

^b Molecular weight (MW) was measured using MALDI-TOF MS.

^c RP-HPLC analysis was used to determine the retention time (RT) of the peptides based on acetonitrile/water/TFA system and C18 column

After confirming the successful synthesis of the three peptides, we proceeded to analyze their secondary structures using circular dichroism (CD) spectroscopy. In DI water, myristoylated KR-12 exhibited a negative peak at approximately 208 nm and a positive peak at approximately 195 nm, suggesting the presence of α -helical structures in these peptides. Importantly, there were no significant differences observed in the CD spectra between myristoylated KR-12 and native KR-12, indicating that the myristoylation did not significantly alter the secondary structure of the peptides. The CD spectra of myristoylated KR-12 were consistent with those of native KR-12 (Fig. 2A). Further analysis confirmed that the secondary structures of myristoylated KR-

12 were nearly identical to native KR-12, with similar percentages of α -helices, β -hairpins, and random coils calculated in DI water (Table S1)

To investigate the self-assembly behavior of the two myristoylated KR-12 derivatives, we utilized transmission electron microscopy (TEM). When Myr-KR-12N or Myr-KR-12C was mixed with DI water at a final concentration of 200 $\mu\text{g/ml}$, spherical formations were spontaneously formed (Fig. 2B). Subsequent analysis using dynamic light scattering (DLS) revealed average hydrodynamic diameter (d_h) values of 136.0 ± 27.7 nm and 202.6 ± 48.6 nm for Myr-KR-12N and Myr-KR-12C, respectively, with relatively low polydispersity index (Fig. 2C-D). Additionally, based on the TEM and DLS results, it was observed that the particle size of Myr-KR-12N was smaller than that of Myr-KR-12C. This difference in particle size could be attributed to the stronger hydrophobicity of Myr-KR-12N compared to Myr-KR-12C. It is known that stronger internal hydrophobicity of self-assembled micelles leads to a stronger stabilizing effect on nanoparticles, resulting in smaller and more uniform particle sizes (Table 1).

2.2 Myristoylated KR-12 potently kills ESKAPE *in vitro*.

Enterococcus faecium, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species, termed as “ESKAPE”, are six highly antibiotic-resistant bacteria that account for the bulk of bacteremia cases and surgical-site infections in hospital settings[21, 24, 25]. A previously reported colony count assay was used to measure the antimicrobial activity of KR-12, KR121, and Myr-KR-12C against ESKAPE members, including gram negative *E. coli* (ATCC 25922), *A. baumannii* (ATCC 17978), *P. aeruginosa* (ATCC 27853), *K. pneumoniae* (ATCC 13883), and gram positive *S. aureus* (ATCC 25923), methicillin resistant *S. aureus* (MRSA, ATCC 43300)[21, 25]. Briefly, bacterial cells (2×10^6 CFU/mL) were introduced to an equivalent volume of double strength (2 $\times$) LB and their growth kinetics were constantly monitored at 37°C on a 96-well plate reader to measure bacterial survival after 2 hours treatment with different doses of peptides. As shown in Fig. 3, Myr-KR-12N and Myr-KR-12C showed more potent killing activities than their parent KR-12 peptide. For example, at 12.5 $\mu\text{g/ml}$ concentration, Myr-KR-12N reduced the survival of *K. pneumoniae* by approximately 5 orders of magnitude while Myr-KR-12C induced a 2 orders of magnitude reduction. By contrast, KR-12 only reduced 1 order of magnitude at the same concentration (Fig. 3E). Overall, the *in vitro* bactericidal activity indicates that the myristoylated Myr-KR-12N and Myr-KR-12C nanobiotic are broad-spectrum nanobiotics and superior in potency to KR-12.

Antimicrobial-resistant bacteria provide a worldwide health issue, increasing death as a result of treatment failure. Some germs can get through the final line of protection, leaving these patients with no available antibiotics. To further investigate the bactericidal activities of myristoylated KR-12, we tested peptide MICs against the ESKAPE members mentioned above, as well as two antibiotic resistant strains including a Vancomycin resistant *S. aureus* (VRSA) strain and a Carbapenem-resistant *A. baumannii* (CRAB) strain. VRSA and CRAB strains were got by a sequential passaging method as previous report[26]. As shown in Table 2, MIC values of Myr-KR-12N and Myr-KR-12C were significantly lower than their parent peptide KR-12 which indicates a more powerful bactericidal activity after our modification. The killing activities of Myr-KR-12N and Myr-KR-12C kept stable when bacteria developed resistance to traditional antibiotics as proved by the same MIC values against *A. baumannii* and CRAB.

Table 2
Antimicrobial activity of KR-12, Myr-KR-12N and Myr-KR-12C.

Antibiotic or AMPs	Minimal Inhibitory Concentrations (MIC) in µg/ml							
	<i>E. coli</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>A. baumannii</i>	<i>P. aeruginosa</i>	MRSA	VRSA	CRAB
KR-12	> 128	> 128	> 128	> 128	64	> 128	> 128	> 128
Myr-KR-12N	8	8	4	8	16	8	8	8
Myr-KR-12C	16	8	8	8	32	8	8	8
Meropenem	0.03125	- ^a	0.0625	0.125	0.125	0.25	-	1
Vancomycin	-	1	-	-	-	-	128	-

-^a Represents not measured.

MRSA: Methicillin-resistant *S. aureus*. VRSA: Vancomycin-resistant *S. aureus*, CRAB: Carbapenem-resistant *A. baumannii*.

2.3 Stability Experiments of Myristoylated KR-12

AMPs often face challenges in salt solutions and protease-rich environments, resulting in a short half-life in vivo and limited bioavailability, thereby impeding their clinical application. Therefore, we evaluated the tolerance of KR-12, Myr-KR-12N nanobiotic and Myr-KR-12C nanobiotic by measuring their MIC against *E. coli* (ATCC 25922), *S. aureus* (ATCC 25923), *K. pneumoniae* (ATCC 13883) and *A. baumannii* (ATCC 17978) in physiological salt and serum conditions. The MIC values of KR-12, Myr-KR-12N, and Myr-KR-12C against the tested bacteria under 150 mM NaCl and 25% serum conditions are presented in Table 3. Among the peptides, KR-12 exhibited no bactericidal activity in both 150 mM NaCl and 25% serum environments. In contrast, Myr-KR-12N and Myr-KR-12C maintained effective antibacterial activity against *E. coli*, *S. aureus*, *K. pneumoniae* and *A. baumannii* under physiological salt and serum conditions, demonstrating the high stability of Myr-KR-12N and Myr-KR-12C.

However, a comparison between Myr-KR-12N and Myr-KR-12C revealed that the antimicrobial activity of Myr-KR-12C was influenced by 150 mM NaCl, whereas Myr-KR-12N was less affected and retained satisfactory bactericidal activity. This observation suggests that Myr-KR-12C's antibacterial activity in physiological salt was lower than that in water, while Myr-KR-12N's activity was practically unaffected by physiological salt compared to water. Additionally, as shown in Table 3, serum had an impact on the antibacterial activity of both Myr-KR-12N and Myr-KR-12C, likely due to the presence of anionic serum proteins such as serum albumin and lipoprotein. These proteins can bind to AMPs and reduce their effectiveness. Nevertheless, Myr-KR-12N exhibited greater tolerance to serum than Myr-KR-12C, indicating its higher stability. These findings from the physiological salt and serum stability experiments align with in vivo studies, which demonstrated better therapeutic efficacy of Myr-KR-12N (Fig. 5A). The enhanced stability of Myr-KR-12N contributes to the improvement of AMPs' half-life and bioavailability in vivo.

Table 3
Stability of KR-12, Myr-KR-12N and Myr-KR-12C.

AMPs	Incubation environment	Minimal Inhibitory Concentrations (MIC) in µg/ml			
		<i>E. coli</i>	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>A. baumannii</i>
KR-12	MIC in water	> 128	> 128	> 128	> 128
	MIC in 150 mM NaCl	> 128	> 128	> 128	> 128
	MIC in 25% serum	> 128	> 128	> 128	> 128
Myr-KR-12N	MIC in water	8	8	4	8
	MIC in 150 mM NaCl	16	8	8	8
	MIC in 25% serum	64	64	64	32
Myr-KR-12C	MIC in water	16	8	8	8
	MIC in 150 mM NaCl	>128	>128	64	8
	MIC in 25% serum	>128	64	>128	32

2.4 Time-kill kinetics

The killing kinetics of KR-12, Myr-KR-12N nanobiotic, and Myr-KR-12C nanobiotic against *E. coli* (ATCC 25922) were examined through time-kill assays. As shown in Fig. 4A, *E. coli* was completely eradicated within 2 hours of incubation with Myr-KR-12N at a concentration of 64 µg/ml. Similarly, complete elimination of *E. coli* was achieved within 4 hours of incubation with Myr-KR-12C at the same concentration. In contrast, KR-12 exhibited a slower bactericidal rate, as *E. coli* was not completely killed until 6 hours at 64 µg/ml, with noticeable regrowth observed after 4 hours of incubation. These findings suggest that Myr-KR-12N and Myr-KR-12C possess rapid bactericidal activity, while KR-12 exhibits a slower killing rate. We speculate that the enhanced bactericidal activity of Myr-KR-12N nanobiotic and Myr-KR-12C nanobiotic can be attributed to their self-assembly into nanoparticles. This self-assembly process increases the contact area between the peptides and the bacterial membrane, leading to rapid accumulation on the surface of the bacterial membrane. Consequently, the peptides insert into the membrane, causing leakage of bacterial contents and ultimately leading to bacterial death.

2.5 Penetrating Test of the Bacterial Outer Membrane (OM)

Gram-negative bacteria possess a more formidable barrier to penetration compared to Gram-positive bacteria, primarily due to the presence of the outer Membrane (OM). The OM of Gram-negative bacteria is predominantly composed of lipopolysaccharide (LPS), an amphiphilic macromolecule that hinders the entry of hydrophobic molecules and is closely associated with bacterial drug resistance, adhesion, and invasiveness. To assess the OM permeability of the *E. coli* (ATCC 25922), we employed 1-N-Phenyl-1-naphthylamine (NPN), a hydrophobic fluorescent dye whose fluorescence is typically quenched in an aqueous environment. When the integrity of the OM is compromised, NPN enters the hydrophobic environment, leading to enhanced fluorescence. Furthermore, the fluorescence intensity is directly proportional to the extent of OM damage.

In our study, the vehicle group treated with sterile water alone exhibited minimal fluorescence enhancement, indicating an intact and unaffected OM with no significant changes in permeability. Conversely, all tested peptides exhibited an increase in NPN fluorescence, indicating enhanced OM permeability (Fig. 4B). Moreover, when comparing peptides at equivalent concentrations, the fluorescence intensity in the Myr-KR-12C-treated group was approximately 1.5 times higher than that in the KR-12-treated group, while the Myr-KR-12N-treated group displayed a fluorescence intensity approximately twice that of the KR-12-treated group. These findings provide evidence that both Myr-KR-12N nanobiotic and Myr-KR-12C nanobiotic possess potent outer membrane-penetrating activity compared to KR-12 alone. Additionally, the penetration ability of Myr-KR-12N was found to be stronger than that of the other peptides.

2.6 Penetrating Test of Bacterial Inner Membrane (IM)

The bacterial inner membrane, also known as the cytoplasmic membrane, is a soft, flexible, semi-permeable membrane composed of phospholipids and proteins. The integrity of the inner membrane is important for the growth and reproduction of bacteria. ONPG (ortho-nitrophenyl- β -galactoside) can be hydrolyzed by β -galactosidase in bacteria to produce colored o-nitrophenol (ONP) which is released to the extracellular in large quantities only when pores are formed in the bacterial inner membrane, so the effect of the peptides on the integrity of the bacterial inner membrane can be judged by the color change in medium.

The KR-12 group reached saturation of ONPG uptake in *E. coli* (ATCC 25922) at approximately 30 min (Fig. 4C), which was not significantly different from the vehicle group, while the Myr-KR-12N or Myr-KR-12C groups resulted in a continued increase in absorbance intensity, indicating that Myr-KR-12N or Myr-KR-12C disrupted the inner membrane of *E. coli* and that this disruption was reversible. These results further demonstrate that the designed Myristoylated KR-12 nanobiotics disrupt the cell membranes of *E. coli* and that Myr-KR-12N has the best disruption effect at the same concentration. The above results showed that Myr-KR-12N nanobiotic or Myr-KR-12C nanobiotic had excellent membrane-disrupting or penetrating ability and could perform antibacterial effects through a membrane-damaging mechanism.

2.7 Flow Cytometry (FCM)

To further assess the damaging effect of KR-12 and Myristoylated KR-12 nanobiotics on bacterial membranes, we employed propidium iodide (PI) staining, which fluoresces upon binding to nucleic acids once the cytoplasmic membrane has been disrupted. Flow cytometry analysis was performed to detect the uptake of PI by *E. coli* (ATCC 25922) treated with the three peptides at the same concentration. In the absence of peptides, only 19.5% of cells exhibited PI fluorescence, indicating the viability of the bacterial membrane (Fig. 4D-G). However, when incubated with Myr-KR-12N and Myr-KR-12C for 1 hour, the percentage of fluorescent bacteria, indicative of membrane disruption, increased to 81.8% and 55.6%, respectively. In contrast, only 40.7% of *E. coli* treated with KR-12 displayed PI staining. These findings indicate that Myristoylated KR-12 nanobiotics possess a more potent ability to disrupt the cytoplasmic membrane of *E. coli* compared to KR-12 alone, with Myr-KR-12N nanobiotic demonstrating the highest disruptive effect.

2.8 Scanning Electron Microscopy (SEM)

The SEM was employed next to analyse the morphological changes in an *E. coli* (ATCC 25922) in response to peptide treatment to better understand the bactericidal mechanisms of Myr-KR-12N and Myr-KR-12C

nanobiotic. Treating *E. coli* for 2 hours with KR-12 at a concentration of 100 µg/ml resulted in little alterations in the bacterial surface, however Myr-KR-12N or Myr-KR-12C nanobiotic resulted in irregularly shaped holes in the membrane (Fig. 4H-K). It seems that the whole membrane including the inner membrane and outer membrane were interrupted. Thus, the capacity of KR-12, Myr-KR-12N nanobiotic, and Myr-KR-12C nanobiotic to produce morphological alterations in *E. coli* appears to be positively associated with their antibacterial efficacy. Given the established mechanisms of Cathelicidin antibacterial action[10], disruption of the bacterial membrane and/or cell wall structure, as demonstrated by SEM data, might be the explanation of death in bacteria exposed with the Myr-KR-12N or Myr-KR-12C nanobiotic.

Overall, the *in vitro* antimicrobial activity findings show that myristoylated KR-12 are broad-spectrum nanobiotics with higher efficacy than their parent peptide KR-12. Myristoylated KR-12 killed pathogens that were resistant to conventional antibiotics, providing potential new choice for treating antibiotic-resistant bacteria.

2.9 Myr-KR-12N rescued mice from lethal sepsis induced by *E. coli* and Myr-KR-12N showed more potent rescuing activity than meropenem (MEM).

Inspired by the potent bactericidal activity *in vitro*, an *in vivo* sepsis animal model was used to test the therapeutic potential of the Myr-KR-12N and Myr-KR-12C. In brief, a fatal dosage of *E. coli* (ATCC 25922) (1×10^7 CFU per animal) was administered intraperitoneally into four groups. The Three treatment groups were given a single dosage of KR-12, Myr-KR-12N, or Myr-KR-12C at 10 µg per mouse after infection, whereas the control group was given a vehicle (equal volume of sterile water). The process of *E. coli* infection-induced sepsis, post-infection treatment, and the 72-hour survival rate are depicted in Fig. 5A. As shown in Fig. 5B, in the vehicle-treated group, none of mice survived beyond 24 hours after infection. Despite the fact that KR-12 therapy increased survival by 20%, there was no statistically significant difference between the vehicle-treated group and the KR-12-treated group. The survival rate was increased to 40% with the Myr-KR-12C nanobiotic therapy. Notably, after therapy with Myr-KR-12N, 100% of the mice survived.

Encouraged by the excellent treating efficiency of Myr-KR-12N in the *in vivo* sepsis animal model. Three groups of mice, each with 5 mice, were intraperitoneally injected with *E. coli* (ATCC 25922) at a fatal dosage of 1×10^7 CFU per mouse to compare the therapeutic effects of Myr-KR-12N with meropenem (MEM), a potent and broad-spectrum antibiotic for clinical use. The groups received a single dose of Myr-KR-12N (10 µg per mouse), MEM (10 µg per mouse), or vehicle after infection. To our surprise, even MEM showed more potent bactericidal activities *in vitro*, proved by much lower MIC values against ESKAPE members as shown in Table 1, only 1 mouse was rescued after treatment with MEM. While, Myr-KR-12N rescue all mice with the same dose (Fig. 5C). To further evaluate the *in vivo* bactericidal activity of MEM, five groups of mice, each with 5 mice, were intraperitoneally injected with *E. coli* at a fatal dosage of 1×10^7 CFU per mouse. These treating groups received four doses of MEM (20 µg, 50 µg, 200 µg, 400 µg per mouse respectively), or vehicle after infection. Figure S7 showed that only 400 µg of MEM rescued all the sepsis mice. These data proved that Myr-KR-12N is superior in treating sepsis mice than the carbapenem antibiotic meropenem (MEM).

Organ bacterial burden after treatment with Myr-KR-12N or KR-12 was then tested to learn more about the processes by which it efficiently treats mice from infection. Mice (n = 4) were infected with a fatal dosage of *E.*

coli (ATCC 25922) (1×10^7 CFU per mouse) and then given a single-dose therapy of either vehicle or 10 μ g of KR-12, Myr-KR-12N, or Myr-KR-12C per mouse. At 6 hours after treatment, organs, blood, and peritoneal lavage fluids (PLFs) were collected for *E. coli* quantification using a colony-count technique. Both Myr-KR-12N and Myr-KR-12C nanobiotics decreased the bacterial load in the sick mice's liver, lung, spleen, kidney, blood, and PLF, as shown in Fig. 6A. However, Myr-KR-12C was much less effective than Myr-KR-12N in reducing the bacterial burden on organs.

The severity of organ failure is directly correlated with the bad prognosis of sepsis. Experimental animals' livers, lungs, and kidneys were fixed before histopathological examination to acquire deeper understanding of organ damage. As seen in Fig. 6B, the vehicle group's lung displayed significant thickening of the respiratory membrane as well as a significant infiltration of inflammatory cells. The liver also displayed bleeding and infiltration, and the kidney displayed hemorrhages, brush border disappearance, and cast formation. While Myr-KR-12N and Myr-KR-12C nanobiotic therapy significantly removed the sepsis-induced tissue damage, KR-12 therapy showed little to no effect in reducing these anomalies.

These *in vivo* findings from the murine sepsis model, taken combined, show that the Myr-KR-12N nanobiotic has greater therapeutic effectiveness in systemic bacterial infections. This effectiveness is better than that of a carbapenem antibiotic meropenem (MEM), making it a promising candidate for clinical application.

2.10 Myristoylated KR12 nanobiotic reduced LPS-induced inflammation *in vitro* and rescued mice from endotoxin sepsis induced by LPS *in vivo*.

Lipopolysaccharide (LPS), a significant causation of inflammation, has been linked to sepsis, and an uncontrolled LPS response plays a key role in this condition. Antibiotic is one of the most important treating strategies in sepsis while some antibiotic therapy may promote the release of inflammatory bacterial products such as lipopolysaccharide (LPS)/endotoxin and has been proved to be linked with some fast clinical deterioration[27–29].

It's expected that the nanosized modified KR-12 with a highly concentrated positive charge and hence has a stronger electrostatic force with the negatively charged LPS, resulting in a superior neutralizing effect. Polymyxin B (PMB) is a cyclic cationic polypeptide antibiotic derived from *Bacillus Polymyxa*[30]. PMB have been discovered to have the ability of binding and neutralizing endotoxin. The mechanism of neutralization is that PMB could bind with high specificity to LPS and lead to its aggregation [31, 32]. As a result, it has been used widely in studies of LPS bioactivity neutralization.

LPS-neutralizing activity such as the inhibition of TNF- α production of AMPs lies in the binding LPS[11]. To investigate the LPS-binding activity of KR-12, Myr-KR-12N and Myr-KR-12C, the binding activity of the peptides to LPS was conducted by chromogenic LAL assay. Binding of the peptides to LPS was determined by measuring the activation of LAL enzyme by the rest free LPS. In the LAL assay, Myr-KR-12N and Myr-KR-12C displayed significant higher LPS-binding activity than KR-12(Fig. 7A).

We investigated the potential anti-inflammatory effects of peptides (KR-12, Myr-KR-12N and Myr-KR-12C) and antibiotics (MEM and PMB) on macrophages in response to LPS. Murine peritoneal macrophages were co-incubated with 100 ng/ml LPS and 5 μ g/ml peptides or antibiotics. After stimulation for 24 h, supernatants

were harvested for IL-6 and TNF- α monitoring. As shown in Fig. 7B, PMB completely abolished the release of IL-6 and inhibited nearly all TNF- α production. IL-6 production was inhibited by 41%, 91% and 72% in response to KR-12, Myr-KR-12N and Myr-KR-12C respectively. What's more, TNF- α production was inhibited by 50%, 86% and 76% in response to KR-12, Myr-KR-12N and Myr-KR-12C respectively. On the contrary, MEM showed nearly no inhibition activity (Fig. 7B-C).

Inspired by the excellent anti-inflammatory efforts *in vitro*, we next induced a mouse model of endotoxin sepsis via intraperitoneal coadministration of LPS (10 ng/mouse) and D-galactosamine (18 mg/mouse), a compound that sensitizes animals to LPS. In the vehicle control group, 90% of the animals died within 24 h after LPS injection. The administration of 10 μ g per mouse KR-12 or meropenem did not statistically differently increased the survival rate, while the survival rate increased to 100% after treatment with the same dose of Myr-KR-12N, Myr-KR-12C, or PMB (Fig. 7D). To further assess the LPS neutralization activities of these peptides, dose of LPS was increased to 50 ng per mouse. As shown in Fig. 7E, the administration of 10 μ g per mouse Myr-KR-12N, Myr-KR-12C and PMB rescued all septic mice. To further analyse the impact of anti-endotoxic sepsis between Myr-KR-12N, Myr-KR-12C, and PMB, a greater dosage of 1 μ g of LPS per mouse was introduced thereafter. As shown in Fig. 7E, Myr-KR-12C has no effect in rescuing mice at this dose. PMB rescued 1 mouse from 10 experiment animals, while Myr-KR-12N rescued 8 from 10 sepsis mice. These findings suggest that these myristoylated KR-12 nanobiotics, particularly Myr-KR-12N, can significantly reduce endotoxin-induced immune responses. Furthermore, Myr-KR-12N outperforms PMB in terms of LPS neutralization activity.

Endotoxin release *in vitro* is dependent on bacterial growth and antibiotic-induced bacterial death. When bacteria are quickly eliminated by antibiotic exposure, endotoxin release over 24 hours is at its lowest; nevertheless, greater release is anticipated when simultaneous growth and antibiotic-induced death take place[29]. We observed no neutralizing effect of MEM on LPS, besides, previous research has reported that meropenem induced high levels of endotoxin release[33, 34], which may partly explains why the MIC for *E. coli* MEM was much lower than that of Myr-KR-12N, but its effectiveness in treating *E. coli* sepsis induced by abdominal infections *in vivo* was much better than that of MEM. It is reported that Cathelicidin and PMB neutralize endotoxins by multifactorial mechanisms including LPS interaction and targeting of host cell membranes[31]. In this work, although Myr-KR-12N was weaker than PMB in neutralizing LPS in cellular assays, its ability to rescue septic mice in an *in vivo* model of endotoxic sepsis was superior to that of PMB. This may be related to their multi-target mechanism of action and further research still needs to be done.

2.11 Myristoylated KR-12 were not toxic *in vitro* and *in vivo*

Side effects caused by AMPs impede their clinical translations. Prior studies indicated that some AMPs could exert cytotoxicity including hemolytic activity[35]. To better understand the toxicity of the nanobiotics, we evaluated the hemolytic activity of KR-12, the Myr-KR-12N nanobiotic, and the Myr-KR-12C nanobiotic *in vitro* using a previously described method[21]. As shown in Figure S8A, neither Myr-KR-12N nor Myr-KR-12C nanobiotics exhibited meaningful hemolytic activity even at relatively high concentrations. To evaluate the safety of the Myr-KR-12N and Myr-KR-12C nanobiotic *in vivo*, we injected mice (n = 5) intraperitoneally with the Myr-KR-12N nanobiotic (100 μ g per mouse) or vehicle and monitored the change in body weight for 7 days. As shown in Figure S9B, no difference in body weight was observed during this period. To further evaluate the *in vivo* toxicity of these peptides, 4 groups of mice (6 mice per group) were subcutaneously injected with 240 μ g

of KR-12, Myr-KR-12N or Myr-KR-12C or PBS every 2 h for 6 times injection according to a previous reported method[36]. At 20 h after the last injection of the drug, liver, kidney and blood were collected from the mice. Serum was taken for liver function (ALT and AST) and kidney function (BUN and CREA) tests. The liver and kidney were fixed in formalin and then subjected to histopathological examination. As shown in Figure S9-10, no biochemical and histopathological abnormalities were observed in KR-12, Myr-KR-12N and Myr-KR-12C groups. All these experiments indicated a low toxicity of Myr-KR-12N and Myr-KR-12C which greatly benefits their clinical translations.

3. Conclusion

In this work, we have developed a novel nanobiotic with dual bactericidal and LPS-neutralization properties. It demonstrated more potent bactericidal activity than meropenem (MEM) in an *in vivo* mouse peritoneal *E. coli* (ATCC 25922) infection model. In mice LPS-induced sepsis model, it exhibited more powerful LPS-neutralizing activity than polymyxin B. LPS-induced excessive inflammation plays a central role in sepsis. The growth of gram-negative bacteria and lysis of bacterial components which can be induced by various antibiotics play key role in LPS-release into bloodstream. In addition to its directly potent bactericidal activity, our nanobiotic could neutralize LPS and both these functions significantly decreased sepsis. This strategy is simple and efficient. It may provide good insights for clinical translation of antimicrobial peptides.

4. Methods

4.1 Materials.

The components of LB, that is tryptone and yeast extract, were purchased from Oxoid Company (England). KR-12, Myr-KR-12N and Myr-KR-12C were purchased from Shanghai Top-peptide Company Limited with a solid phase peptide synthesis method. MHB broth was purchased from Oxoid Company (England). The molecular masses of the synthesized peptides were ascertained by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Purity of the peptides were verified by reversed-phase high-performance liquid chromatography (RP-HPLC).

4.2 Circular dichroism spectroscopy

The circular dichroism (CD) spectroscopy of the peptides was obtained at room temperature with a CD spectrometer (Chirascan QCD, Applied Photophysics Ltd.) equipped with a quartz cuvette with a 1 mm path length. The spectra were recorded at wavelengths ranging from 190 to 260 nm at a peptide concentration of 200µg/ml in DI water. An average of three scans was recorded for each peptide, subtracting the averaged spectrum of DI water (baseline) to eliminate solvent contributions. Finally, the CD curves were plotted according to the following formula:

$$\theta = (\theta_{\text{obs}} \times 1000)/l.c$$

θ is the mean residue ellipticity ($\text{deg}\cdot\text{cm}^2\cdot\text{mol}^{-1}$); θ_{obs} is the observed ellipticity after correction by the DI water at a given wavelength (mdeg); c is the peptide concentration; l is the path length (mm).

4.3 Transmission Electron Microscopy (TEM).

The morphologies of the peptide micelles were observed under a JEM-1010 electron microscope (JEOL, Japan) using an acceleration voltage of 80 kV. Samples were prepared by dropping peptides (200 µg/ml) solutions onto a 230-mesh copper grid coated with carbon. A few minutes after deposition, the surface water was removed with filter paper, and then the samples were dried at room temperature. Negative staining was performed using a 2 wt % aqueous uranyl acetate solution followed by air drying and TEM analysis was performed thereafter.

4.4 Dynamic light scattering (DLS)

To reveal the average particle size, a Malvern Nano-ZS90 instrument (Malvern, UK) was used to monitor the number distribution of peptides (100 µg/ml) and hydrodynamic diameters (dh) according to the DLS principle. The peptide solutions (1 mL) were placed in cuvette. The refracted index and viscosity of pure water were used as the solvent settings, while the refracted index of the solute was set to be the same as that of the proteins. The temperature was maintained at room temperature and the solution was equilibrated for 5 min before data collection. Three measurements were performed for each sample. The data were analyzed automatically with the software to generate the number-based size distribution of the complexes.

4.5 Bacteria.

Bacterial strains, namely *S. aureus* (ATCC 25923), MRSA (ATCC 43300), *E. coli* (ATCC 25922), *K. pneumoniae* (ATCC 13883), *P. aeruginosa* (ATCC 27853), and *A. baumannii* (ATCC 17978) were obtained from American Type Culture Collection (ATCC).

Vancomycin resistant *S. aureus* (VRSA) and Carbapenem-resistant *A. baumannii* (CRAB) were induced with a previously reported method[26]. Briefly, MICs of bacteria (*S. aureus* ATCC 25923 or *A. baumannii* ATCC 17978) were calculated according to CLSI guidelines. Thereafter, survival cells at 0.5-fold MIC were used to inoculate the culture tubes of the next passage. This was repeated for more than 20 passages. The MIC fold changes were recorded and calculated to indicate the development of drug resistance. VRSA were obtained with a final MIC value of vancomycin against *S. aureus* (ATCC 25923) reached 128 µg/ml. CRAB was obtained with a final MIC value of imipenem against *A. baumannii* (ATCC 17978) reached 12.5 µg/ml.

4.6 Antibacterial Assay *in vitro*.

The antibacterial activity was determined with a virtual colony-count assay as previously described[21, 25]. In brief, single colonies from LB agar plates were incubated in 5 mL of LB. After growing for 4–6 h at 37°C, 200 rpm in an incubator, the bacteria were washed three times. After incubation with the AMPs or vehicle for 2 h, a 2-fold concentration of LB was added and bacterial growth was monitored at 600 nm using a Molecular Devices SPECTRA MAX 190 microplate reader (Sunnyvale, CA, USA.) running SoftMax Pro software (version 6.5).

The minimal inhibitory concentrations were determined according to CLSI guidelines. Briefly, single colonies of bacteria from MHB agar plates were incubated in 5 mL of MHB medium overnight at 37°C, 200 rpm in an incubator. Bacterial suspension at 2×10^6 CFU/ml were plated into sterile 96-well plates (Corning, USA) with 50 µl/well, post-incubated with two-fold serial dilutions of antibiotics or peptides (50 µl/well), supplemented with 100 µL 2× MHB medium for bacterial culture. The 96-well plates were incubated at 37°C and the OD600 monitored continuously using a Molecular Devices SPECTRA MAX 190 microplate reader (San Jose, CA, USA).

The minimal inhibitory concentration (MIC) for each bacterial species was taken as the lowest concentration of antibiotics or peptides that reduced growth (OD600) by more than 90% when compared to the positive growth control.

4.7 Stability Test.

The stability of the peptides was assessed by measuring the MIC of the peptides in physiological concentrations of salt solutions or serum. For stability assay in salt solutions, 50µL of peptides were diluted to different concentrations in 96-well plates with a final concentration of 150 mM NaCl. For stability assay in serum, 50µL of peptides were diluted to different concentrations in 96-well plates with serum. *E. coli* (ATCC 25922) or *S. aureus* (ATCC 25923) was used with the above test, and subsequent steps were corresponding to the MIC assay.

4.8 Time-kill kinetics.

The time-kill kinetics of the three peptides against *E. coli* (ATCC 25922) was measured following a previously described method. An equal volume of bacterial suspension at 1×10^6 CFU/ml was mixed with KR-12, Myr-KR-12N or Myr-KR-12C (64 µg/ml). Samples were taken at 0, 30, 60, 120, 240 and 360min, diluted, and plated onto LB agar plates. The number of viable colonies was determined by plate counts after incubation at 37°C for 16–18 h.

4.9 Outer membrane (OM) permeability assay.

The outer membrane penetrating activity of the three peptides was investigated with fluorescent dye 1-N-phenylnaphthylamine (NPN). The same concentrations of KR-12, Myr-KR-12N and Myr-KR-12C (64µg/ml) were first prepared with sterile water. *E. coli* (ATCC 25922) grown to the logarithmic phase at 37°C were harvested by centrifugation at 4000 rpm for 10 min. The bacteria were washed three times and resuspended to OD600 = 0.2. Bacteria medium (100 µL/well), 40 µM NPN (50 µL/well) and the peptides solution (50 µL/well) were added into sterile 96-well plates and mixed thoroughly. The final concentrations of peptides were 16 µg/ml, and the vehicle group contained bacteria medium (100 µL/well), 40 µM NPN (50 µL/well), and sterile water (100 µL/well). The fluorescence intensity (excitation wavelength Ex = 350 nm, emission wavelength Em = 420 nm) was determined respectively every 30 s for 10 min.

4.10 Inner Membrane (IM) permeability assay.

Orthonitrophenyl-β-galactoside (ONPG) was used to evaluate the penetrating activity of the peptides to the bacterial inner membrane. The same concentrations of KR-12, Myr-KR-12N and Myr-KR-12C were first prepared with sterile water. *E. coli* (ATCC 25922) grown to the logarithmic phase at 37°C were harvested by centrifugation at 4000 rpm for 10 min. The bacteria were washed three times and resuspended with ONPG solution (1.5 mM) and added to sterile 96-well plates (50 µL/well). The peptides were added to 96-well plates (50 µL/well), sterile water (50 µL/well) was used as the vehicle group. The absorbance intensity was determined with a microplate reader every 5 min for 90 min.

4.11 Flow cytometry

The membrane damage was determined by flow cytometry. Briefly, *E. coli* (ATCC 25922) grown to the logarithmic phase at 37°C were harvested by centrifugation at 4000 rpm for 10 min. The bacteria were washed

three times and resuspended to 1×10^6 CFU/ml. The same concentrations of KR-12, Myr-KR-12N and Myr-KR-12C (64 µg/ml) were mixed thoroughly with the bacteria medium and then incubated at 37°C for 2 h. Then, the above mixed solution was centrifuged and washed, and propidium iodide solution (PI solution, the final concentration was 50 µg/mL) was added and incubated at 37°C in the dark for 15 min, centrifuged, washed, and resuspended with PBS (400 µL). The results of FCM were recorded with FCM (BD Canto II) at a laser excitation wavelength of 488 nm.

4.12 Scanning Electron Microscopy (SEM).

The morphology of *E. coli* (ATCC 25922) after treatment with peptides was observed under a SU-8010 field-emission SEM (Hitachi, Japan). Bacteria were incubated with different peptides (100 µg/ml) or vehicle for 2 h. The solution was centrifuged at 4000 rpm for 10 min and washed with PBS two times. A 2.5% glutaraldehyde solution was added for fixation overnight at 4°C. Then, the bacterial were washed with PBS three times and then incubated with 1% OsO₄ in PBS for 1.5 h. After washing another three times with PBS, dehydration was performed using a graded ethanol series. After drying in a LEICA EM CPD300 critical point dryer (LEICA, Germany), the samples were placed on carbon tape adhere to an aluminum stud and coated with platinum prior to SEM analysis.

4.13 Hemolysis Assay.

Fresh mouse RBCs were washed three times with cold PBS. A 20 µL of RBC suspension in PBS (4% in volume) was placed in an Eppendorf (EP) tube with 200 µL of peptides at different concentrations, PBS, or 1% Triton X-100. These tubes were incubated at 37°C and were shaken in a Thermomixer Comfort instrument (Eppendorf, Germany) for 1 h. After incubation, the tubes were centrifuged at 350 g for 5 min. Then, 100 µL of the supernatants mixed with 100 µL of PBS was transferred to 96-well plates and hemoglobin release was monitored at 576 nm using a Molecular Devices SPECTRA MAX 190 microplate reader (Sunnyvale, CA, USA). An RBC suspension in PBS was used as a negative control. The absorbance of the wells containing 1% Triton X-100 was taken as 100% hemolysis. The percentage of hemolysis was calculated using the following formula: hemolysis (%) = [(O.D.576 nm in the peptide solution - O.D.576 nm in PBS)/(O.D.576 nm in 1% Triton X-100 solution - O.D.576 nm in PBS)] × 100. The tests were repeated for five times, and the data are expressed as the mean and standard deviation of five replicates.

4.14 *In vivo* Studies.

All animals received care according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. They were housed under aseptic conditions with a 12 h day and night cycle and given an autoclaved rodent diet and sterile water. The animal experiments were approved by the Animal Care and Use Committee of Zhengzhou University. All animals used in this experiment were bought from Beijing HFK Bioscience Co. Ltd. (Beijing, China).

4.15 *E. coli* (ATCC 25922) Sepsis Model of C57BL/6 Mice.

5 mice (6–8 weeks old) in one group were lethally challenged with 1×10^7 CFU of *E. coli* (ATCC 25922) intraperitoneally. Immediately after the challenge, mice were intraperitoneally injected with 10 µg of KR-12 or the Myr-KR-12N nanobiotic or the Myr-KR-12C nanobiotic or vehicle or different doses of meropenem. Mortality was assessed every 12 hours for 7 days. To determine the bacterial burden, another 4 groups of 4 mice (6–8

weeks old) were lethally challenged with 1×10^7 CFU of *E. coli* (ATCC 25922) intraperitoneally. Immediately after the challenge, the mice were intraperitoneally injected with 10 µg of KR-12 or Myr-KR-12N nanobiotic or Myr-KR-12C nanobiotic or vehicle. At 6 h after injection, the mice were given anesthesia, and their liver, lung, spleen, kidney, blood, and PLFs were collected. PLF was collected by intraperitoneal injection of 4 mL of cold PBS and extraction after gently shaking the entire body as described previously.[21] Part of these organs were fixed with 4% formalin and were used for morphological studies through H&E staining. The rest of the liver, lung, spleen, and kidney tissues were weighted separately, and 10 mL of cold PBS was added for every 1 g of tissue, followed by homogenization with an IKA T10 basic homogenizer (IKA, Germany). Homogenate or dilutions (10 µL) were placed on an LB agar plate. The number of CFU was counted after incubation overnight at 37°C and was expressed as 1 g CFU/g for tissues and 1 g CFU/mL liquid for blood and PLF. The data are expressed as the geometric mean and standard deviation of four values obtained from 4 mice.

4.16 Animal Model of Endotoxin sepsis.

Seven-week-old male C57BL/6 mice were distributed randomly in experimental groups (n = 10 per group). Endotoxemia was induced by intraperitoneal coadministration of LPS and D-galactosamine (18 mg/mouse), a compound that sensitizes animals to LPS[31, 37]. LPS was dissolved in endotoxin-free saline and injected at 10 ng/mouse (LD90) or 50 ng/mouse or 1 µg/mouse. Immediately after the LPS injection, animals received injection of 10 µg peptide or PMB in 150 µl of pyrogen-free saline at a different peritoneal site. Survival was monitored at 12 h intervals for 72 h. Survival plots were compared statistically using the Kaplan-Meier survival analysis. All P values represent comparisons of mortality data from the same experiment.

4.17 Measurement of LPS Neutralization

Peptides neutralizing endotoxin were evaluated by chromogenic limulus lysate (LAL) analysis according to the manufacturer's instructions, with some modifications. Constant endotoxin (1 EU/ml) was cultured in different media. Different concentrations of peptides (1 µg/ml, 10 µg/ml, 100 µg/ml) in the wells of pyrogen sterile microtiter plates were heated at 37°C. Then, 50-µl aliquots of concentrated LAL reagent were added and incubated at 37°C for 10 min. The 100-µl color matrix was yellow. The reaction was stopped by adding 25% hydrochloric acid, and the absorbance was measured at 545 nm.

4.18 Stimulation of mouse Macrophages by LPS.

Mouse peritoneal macrophages were obtained after intraperitoneal injection of sterile thioglycolate medium (3%; EMD Chemicals, Darmstadt, Germany) for 72 h. The cells were cultured in Dulbecco's Minimum Essential Medium (DMEM) containing 10% fetal calf serum (Life Technologies, Grand Island, NY) 100 U/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Grand Island, NY) at 37°C in the presence of 5% CO₂ for 24 h. For stimulation experiments, macrophages were seeded with serum free DMEM medium after washed twice with PBS and then were co-incubated with peptides/antibiotics and LPS for 24 h at 37°C. Cell-free supernatants were analyzed for TNF-α and IL-6 using commercially available ELISA kits (MULTISCIENCES biotech, Co., Ltd, Hangzhou, China).

4.19 Statistical analysis.

Statistical analysis was performed using an independent sample t test or a one-way analysis of variance (ANOVA) using a Bonferroni test to determine statistical differences between groups. The significance level

was set as follows: # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. All statistical data analyses were performed using GraphPad Prism software (version 9.0). All experiments were performed in triplicate, except where indicated.

Declarations

5.1 Corresponding authors

Correspondence Ruyi Lei, Chao Lan, Changju Zhu or Xiangming Fang.

5.2 Ethics approval and consent to participate

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals Center of Zhengzhou University and approved by the Animal Ethics Committee of Laboratory Animals Center of Zhengzhou University.

5.3 Consent for publication

Not applicable.

5.4 Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

5.5 Competing interests

The authors declare no competing interests.

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5.7 Authors' contributions

#R. Lei, Y. Sun, T. Zhu and C. Yang contributed equally to this work.

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Figures

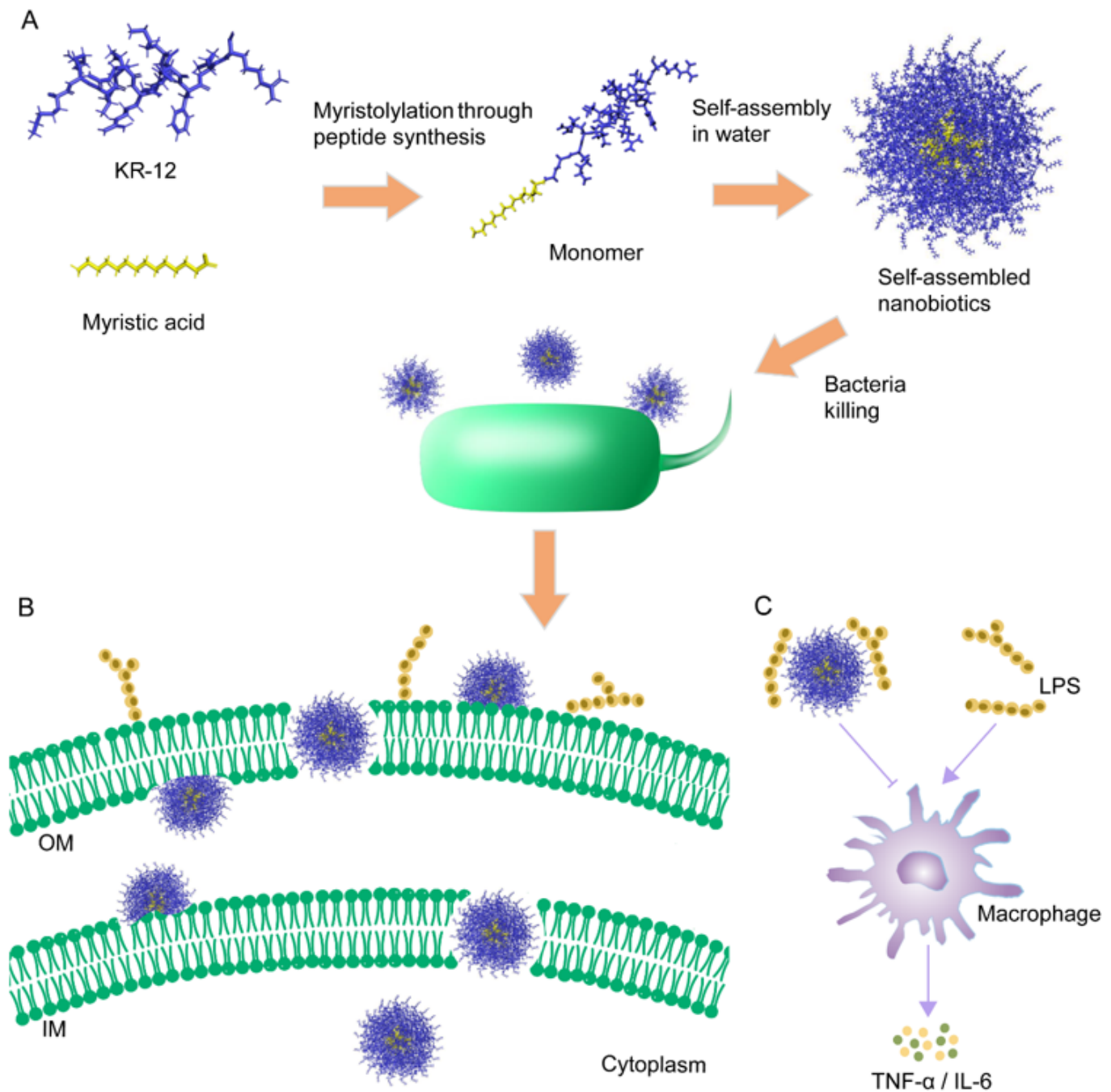


Figure 1

(A) schematic illustration of KR-12 through peptide myristoylation at the N- or C- terminus and sequential nanoassembly of myristoylated KR-12 for generating nanobiotics. (B) Antibacterial mechanism of Myristoylated KR-12 nanobiotics. Myristoylated KR-12 nanobiotics can exert a membrane-penetrating effect by binding to a bacterial outer membrane (OM) or inner membrane (IM) and forming pores in the bacterial membrane. (C) In addition, Myristoylated KR-12 nanobiotics has a highly concentrated positive charge and thus has a stronger electrostatic force with the negatively charged Lipopolysaccharide (LPS) produced by bacteria, resulting in a superior neutralizing effect that inhibits the production of large amounts of pro-inflammatory factors such as TNF- α and IL-6. Throughout the antibacterial process, Myristoylated KR-12

nanobiotics can exert antibacterial activity through both membrane-penetrating and immunomodulatory mechanisms of action.

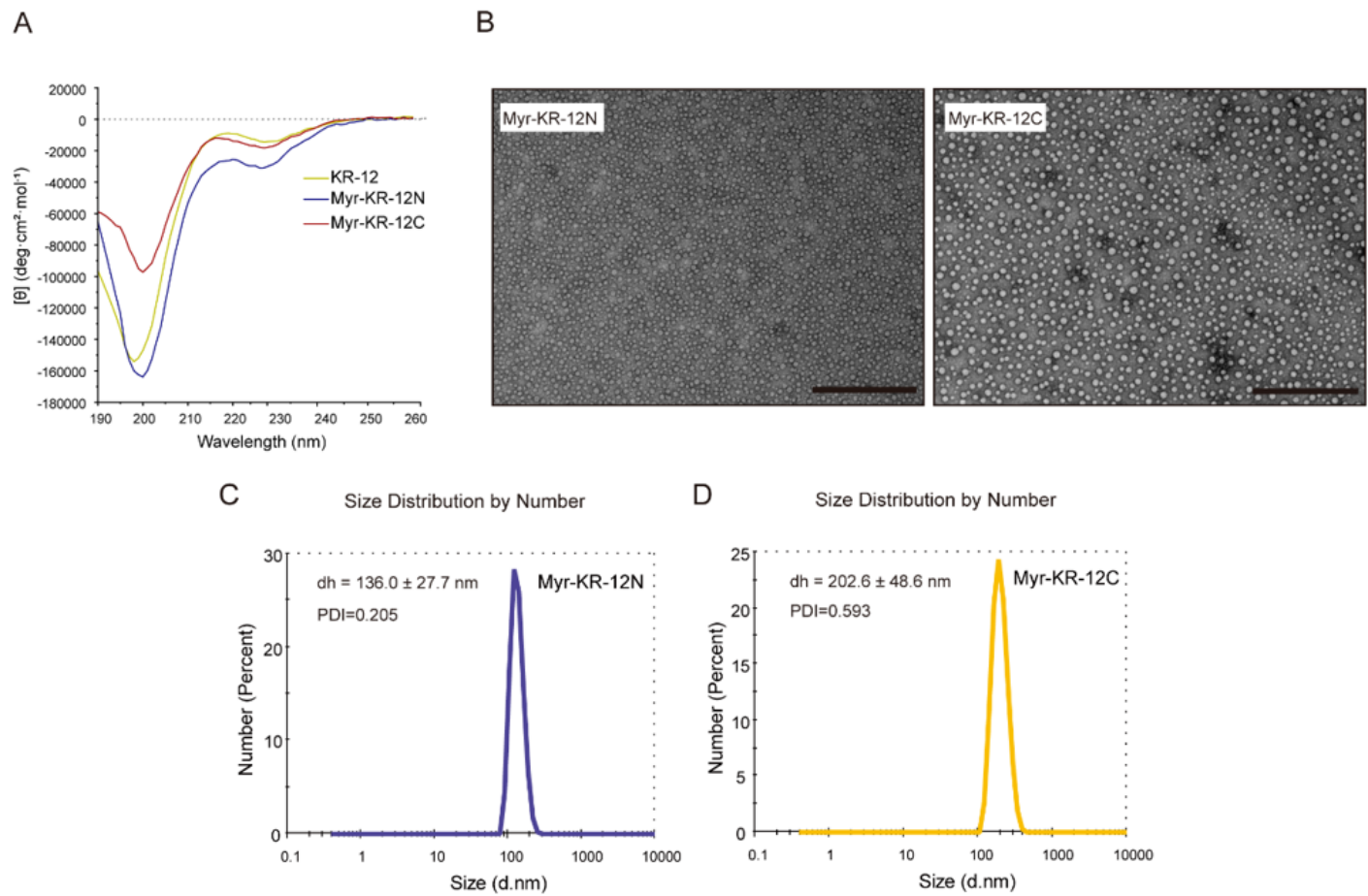


Figure 2

(A) Circular dichroism (CD) spectroscopy of KR-12, Myr-KR-12N and Myr-KR-12C which were dissolved in DI water. (B) Transmission electron microscopy (TEM) images of Myr-KR-12N- and Myr-KR-12C-assembled nanoparticles in DI water. Scale bars, 500 nm. (C-D) Size distribution of Myr-KR-12N and Myr-KR-12C nanoparticles determined by dynamic light scattering (DLS).

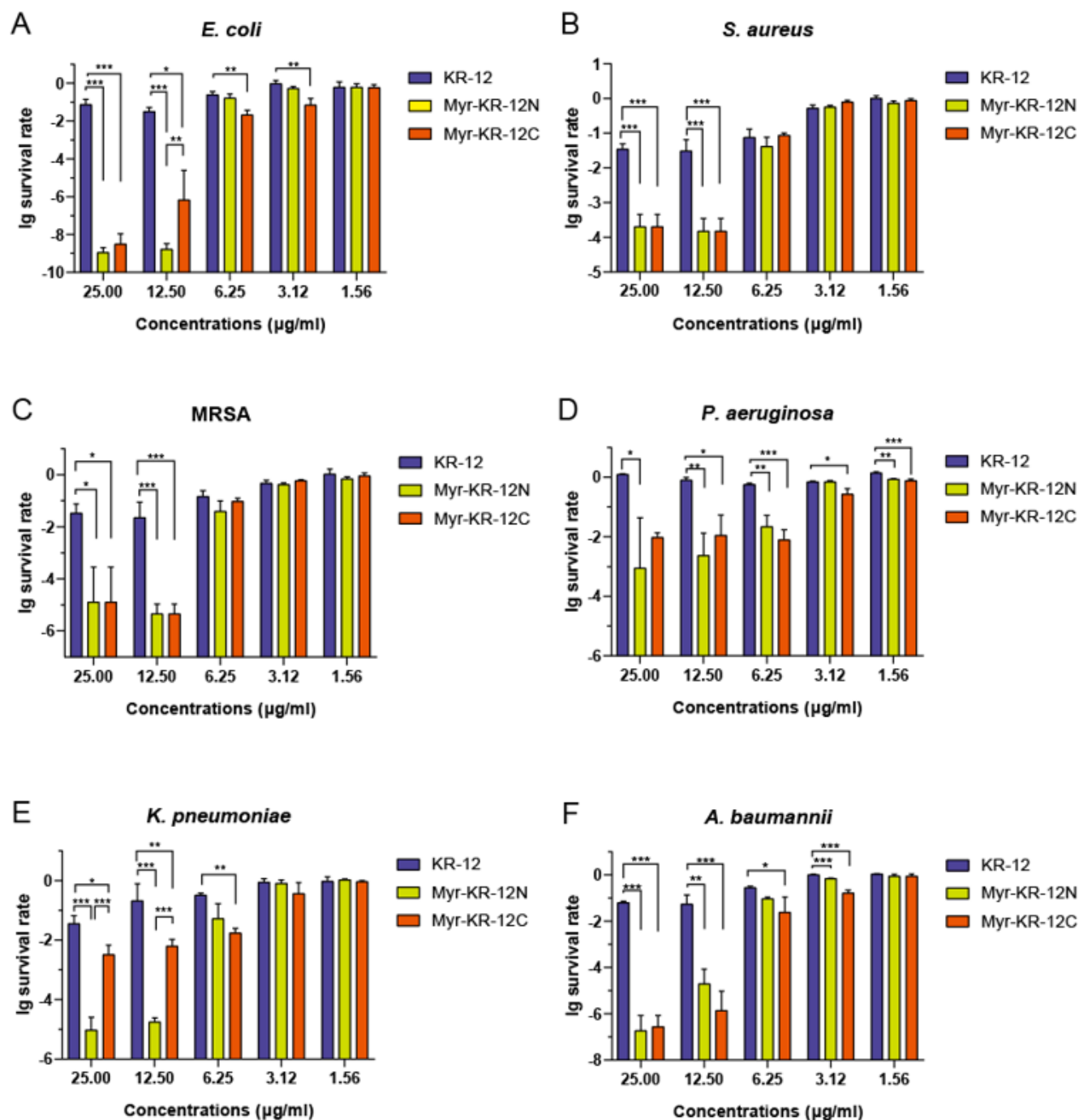


Figure 3

Antimicrobial activity of KR-12, Myr-KR-12N and Myr-KR-12C. (A-F) Dose dependent survival of *E. coli*, *S. aureus*, Methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* treated with KR-12, Myr-KR-12N and Myr-KR-12C. Before being cultured in LB broth, bacteria (1×10^6 CFU/ml) were incubated with these peptides at concentrations ranging by a two-fold increase from 0.78 to 25 µg/ml. The percent bacterial survival (on a logarithmic scale) was calculated as the colonies from treatment wells in comparison to those from vehicle wells. Data from 1.56 to 25 µg/ml are shown. Data from three independent experiments are shown as the mean $\pm$ SD, and statistical significance was calculated using one-way ANOVA and Bonferroni's multiple comparisons test with the following p values: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

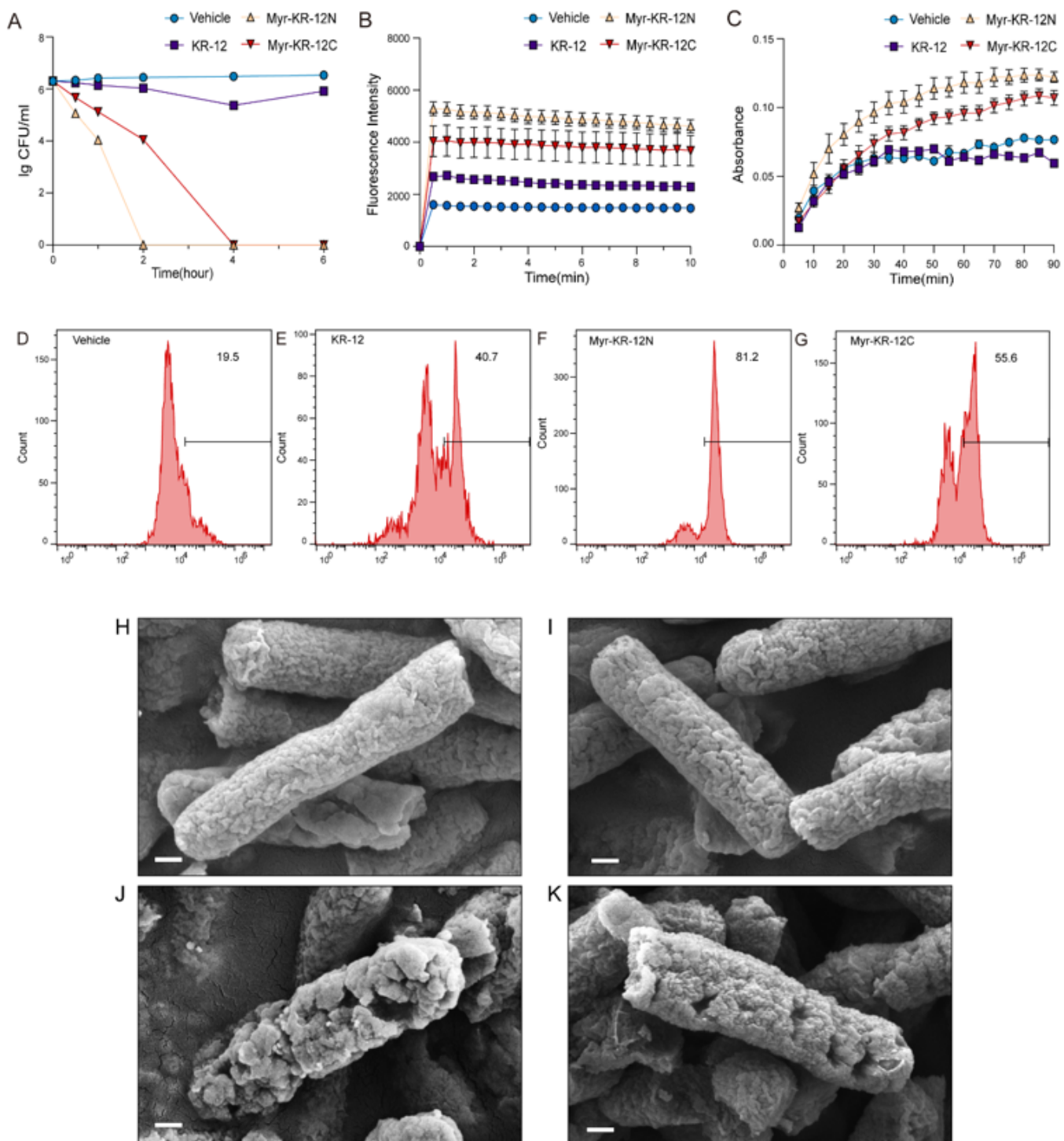


Figure 4

(A) Time-kill kinetics of KR-12, Myr-KR-12N and Myr-KR-12C at 64 $\mu\text{g/ml}$ against *E. coli* (ATCC 25922); (B) Outer membrane penetrating ability of *E. coli* (ATCC 25922) was assessed using an NPN uptake assay; (C) Inner membrane penetrating ability of *E. coli* (ATCC 25922) was assessed using ONPG solution; (D-G) Flow cytometric analysis. *E. coli* (ATCC 25922) were treated with the peptides, and bacterial fluorescence was analyzed by flow cytometry, Vehicle(D) (No peptide, 19.5%); KR-12(E) (40.7%); Myr-KR-12N(F) (81.2%); Myr-KR-12C(G) (55.6%); (H-K) SEM images of *E. coli* after treatment with Vehicle(H), KR-12(I), Myr-KR-12N (J) and Myr-KR-12C (K) for 2 h. Scale bars are 200 nm in length. SEM, Scanning electron microscopy.

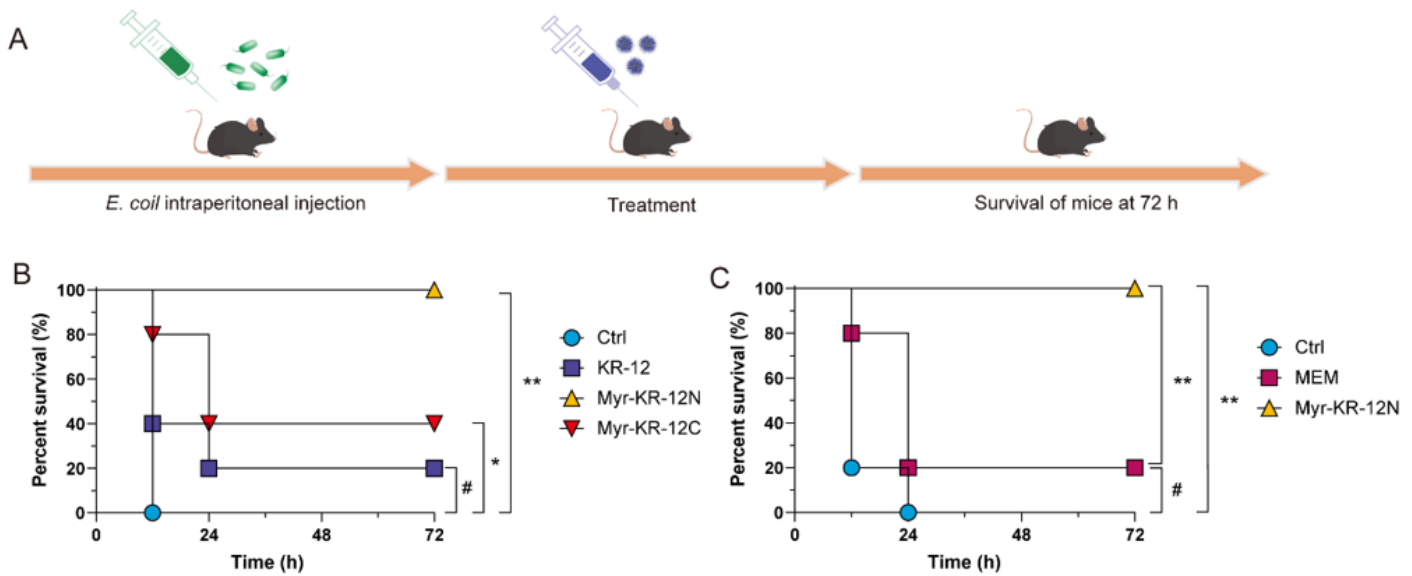


Figure 5

Effectiveness in therapy of KR-12 and its variations in sepsis. (A) Scheme of treatment and 72h survival rate of *E. coli*-infected septic mice (6-8-week-old male C57BL/6J). 10 mice per group were intraperitoneally challenged with 1×10^7 CFU of *E. coli* (ATCC 25922), and either vehicle, KR-12, Myr-KR-12N, Myr-KR-12C (10 μ g per mouse) (B), or vehicle, Myr-KR-12N and MEM (10 μ g per mouse) (C) were then administered as single-dose treatments. The mice were then followed up with a Kaplan-Meier survival analysis. Statistical analysis was calculated using one-way ANOVA and Bonferroni's multiple comparisons test using the following p values: # $p > 0.05$, * $p < 0.05$, ** $p < 0.01$.

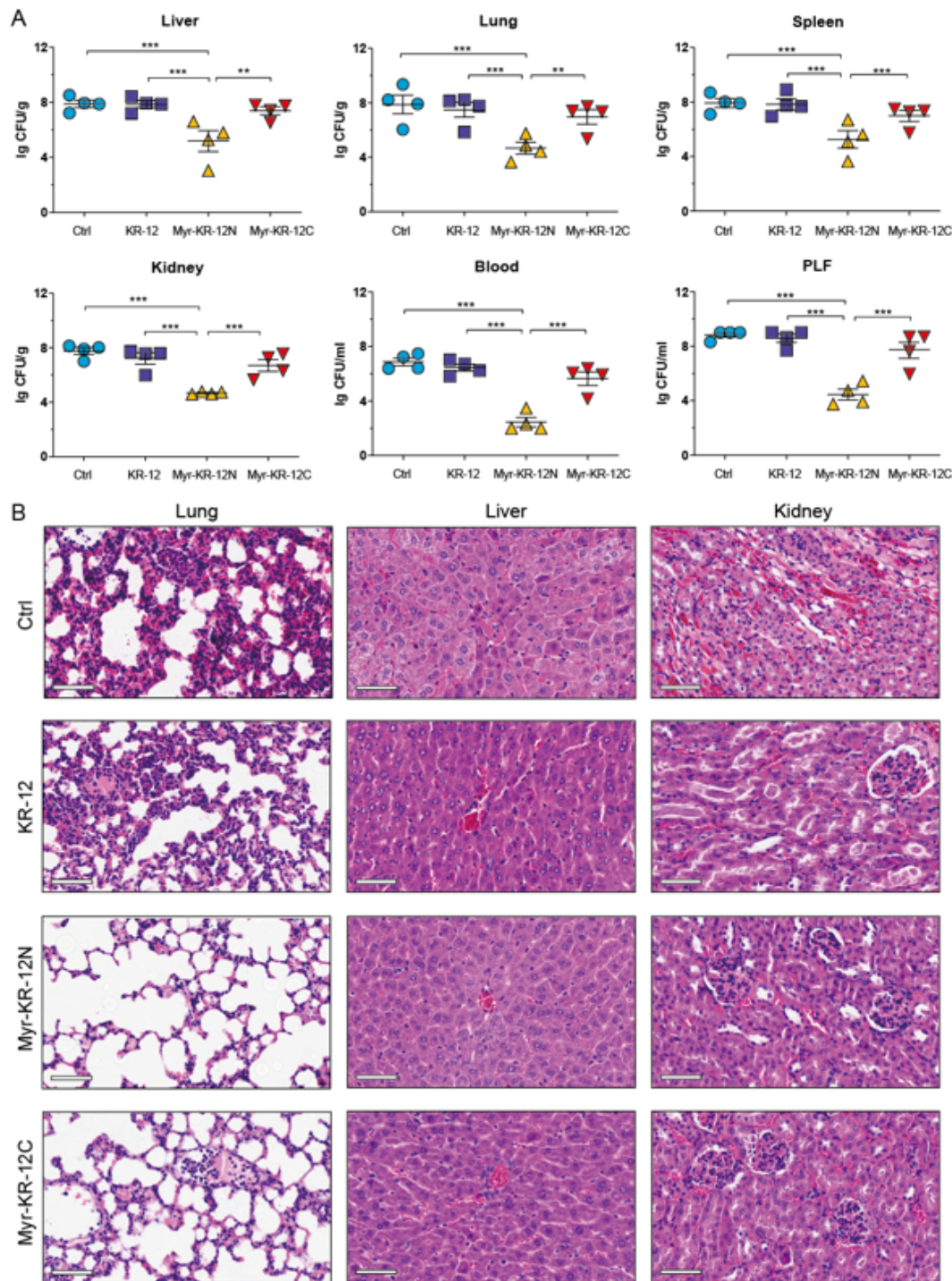


Figure 6

Bacterial burden and organ injury of sepsis mice. After being intraperitoneally challenged with 1×10^7 CFU of *E. coli*, four groups of mice ($n=4$ per group) received either vehicle or a single dose of $10 \mu\text{g}$ of KR-12, Myr-KR-12N, or Myr-KR-12C. Samples from harvested liver, lung, spleen, kidney, blood, and peritoneal lavage fluids (PLF) were taken at 7 hours after treatment for bacterial quantification (A) and histological H&E staining (B). Data are presented as the geometric mean $\pm$ SEM. Statistical significance was calculated using one-way ANOVA and Bonferroni's multiple comparisons test with the following p values: ** $p < 0.01$, *** $p < 0.001$.

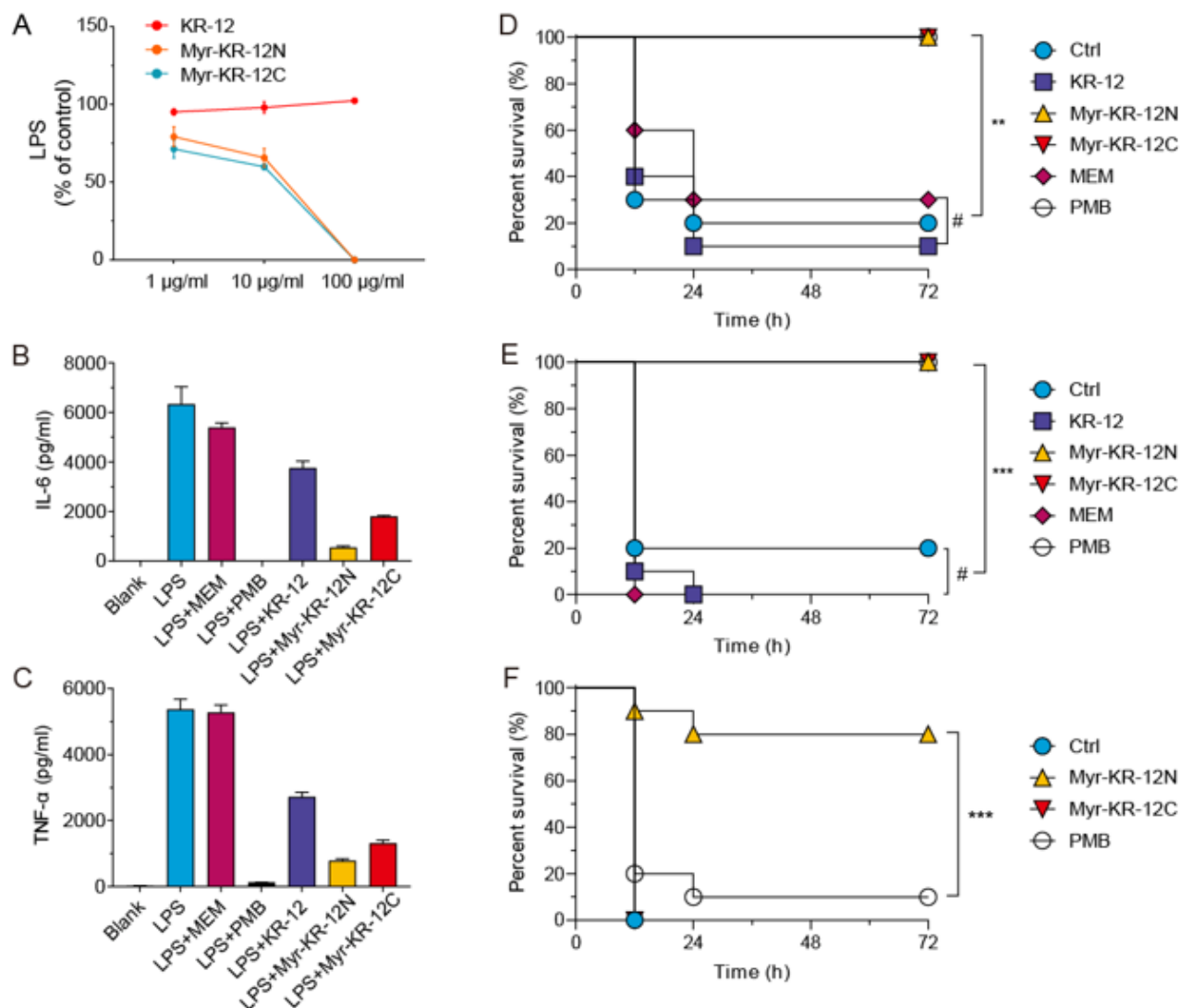


Figure 7

LPS-binding activity and anti-inflammation efforts of KR-12, Myr-KR-12N, Myr-KR-12C and therapeutic efficacy in endotoxin sepsis. (A) Ability of the peptides to bind with lipopolysaccharide (LPS) was evaluated by Limulus amoebocyte lysate (LAL) according to manufacturer's instructions. (B-C) Murine peritoneal macrophages were co-incubated with 100 ng/ml LPS and 5 µg/ml peptides or antibiotics. After stimulation for 24 h, supernatants were harvested for IL-6 (B) and TNF-α (C) monitoring. The data are shown as the means ± SD of 3 independent experiments. (D-F) Kaplan-Meier survival analysis of galactosamine-sensitized mice, 10 in each group, intraperitoneally challenged with 10 ng (D), 50 ng (E) or 1 µg (F) of LPS and then administrated a single-dose treatment of vehicle, KR-12, Myr-KR-12N, Myr-KR-12C, MEM or PMB (10 µg per mouse). Statistical analysis was calculated using one-way ANOVA and Bonferroni's multiple comparisons test using the following *p* values: # *p*>0.05, ** *p*<0.01. *** *p*<0.001.

Supplementary Files

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